PATENT SPECIFICATION (11) 1346061

1346061

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(19)

(54) IMPROVEMENTS IN OR RELATING TO MICROORGANISMS

(71) We, RANKS HOVIS McDOUGALL LIMITED, of Millocrat House, 53 Eastcheap, London, E.C.3, a British Company, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention is for improvements in or relating to microorganisms and has particular reference to a new strain of *Fusarium graminearum* Schwabe which has been isolated from a soil sample.

The new strain of *Fusarium graminearum* Schwabe may be employed in processes for the production of an edible protein containing substance described in our Applications Nos. 53312/66, Serial No. 1,210,356 and 30584/70 and Cognate No. 10466/71, Serial No. 1,346,062 comprising incubation with aeration in a non-toxic carbohydrate containing substrate of vegetable or animal origin employing either batch of continuous culture.

The invention provides the new strain of *Fusarium graminearum* Schwabe deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 145425 and variants and mutants thereof.

The invention further provides the following variants:—

I—7 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154209.

I—8 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154211.

I—9 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154212.

I—15 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154213.

I—16 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154210.

It is well within the knowledge of those skilled in the art to produce variants and mutants of the strain I.M.I. 145425 which show slightly altered properties without affecting the suitability of the strain to produce an edible protein containing substance. The preparation and use of such mutants and variants from the strain I.M.I. 145425 is therefore within the scope of the invention.

The new strain of *Fusarium graminearum* Schwabe may be cultivated in the usual media known to be suitable for the cultivation of *Fusarium* species. For general cultivation malt extract agar has been found convenient and satisfactory. A suitable liquid nutrient medium may contain molasses as the major source of assimilable carbon and ammonium sulphate as the major source of assimilable nitrogen.

According to the invention in another aspect there are provided fungal cultures containing a strain of *Fusarium graminearum* Schwabe, I.M.I. 145425 (or a mutant or variant thereof) in a culture medium, and methods for cultivating the same strain, in which this strain is present in a culture medium containing or being supplied with nutrients or additives necessary for the sustenance and multiplication of the strain, the medium having a pH between 3.5 and 7 and the temperature of the medium being maintained at a precise value within the range of between 25 and 34° C.

Our new strain of *Fusarium graminearum* Schwabe, I.M.I. 145425 is non-pathogenic to wheat. It has the following morphological characteristics:—

Media	Potato Sucrose Agar 250 grams of potatoes washed and diced, placed in pressure cooker 15 lbs. square inch for 15 minutes. The decoction is then squeezed through two layers of muslin. 2% of Glucose and 2% of Agar are added to the turbid filtrate and the medium autoclaved and dispersed.	Czapek-Dox (Modified) Agar (Oxoid) "Oxoid" is a Registered Trade Mark
Growth conditions		25° C. several weeks
Rate of growth: 4.0 cm. in 3 days		3.0 cm. in 3 days

Character of growth:

Floccose, spreading colonies with white aerial mycelium. Substratum on PSA greyish rose with patches of crimson to yellow. Tendency to be somewhat paler on CDA. Occasionally deep red pigment produced, particularly on ageing. After one to two weeks the aerial mycelium tends to become brown and collapse. The colony then becomes rather slimy as sporodochia are formed the colour being pink to brown on PSA and salmon pink on CDA.

No exudate is formed and pigment formation tends to follow the mycelium colour.

Conidia:

Microconidia not produced by this organism. Macroconidia produced from single lateral phialides or multibranched conidiophores with short phialides. In older cultures the conidiophores aggregate to form sporodochia, particularly on CDA. The conidia vary from falcate to curved fusoid dorsi-ventral, septation varying from 3 to 5, commonly 5 in younger cultures. Spore size varies from 25—50μ×2.5μ—4.0μ. The foot cell is often pedicellate, particularly in the longer 5 septate spores. Swollen cells occur in the mycelium and occasionally chlamydospores occur intercalary, singly or in chains.

Following is a description by way of example of the preparation of variants (or isolates) of *Fusarium graminearum* Schwabe I.M.I. 145425 and their morphological characteristics.

EXAMPLES 1 to 5.

Isolates obtained from continuous culture

Fusarium graminearum Schwabe I.M.I. 154209 to 154213 were selected from malt extract agar plates inoculated with broth from a fermenter growing *Fusarium graminearum* Schwabe 145425 on a glucose/salts medium at 30° C. under continuous culture conditions with carbon limitation at a dilution rate of 0.1—0.15 hr^{-1}. The fermenter was sampled at approximately 100 hour intervals to assess the population of variants and the isolates I.M.I. 154209 to 154213 were taken from the plates prepared from the 1100 hour sample. These isolates are representative of the major types of morphological variant produced during the fermentation. Under these conditions outlined in this example the variants begin to appear on plates from 800 hours onwards. They are continually produced from this time onward and the population slowly changes as regards the percentage of each type. The fermenter conditions are as follows:

	Culture medium	%
Solution 1	Glucose	3.0
	Ammonium sulphate	0.25
	Potassium di-hydrogen phosphate	0.30
	Magnesium sulphate	0.025
	Antifoam, polypropylene glycol 2000; sterilised at pH 3.0 for 30 mins. at 15 p.s.i.g	0.01
Solution 2	$MnSO_4$ $4H_2O$	0.0005
	$FeSO_4$ $7H_2O$	0.0005
	$ZnSO_4$ $7H_2O$	0.0005
	$CoCl_2$ $6H_2O$	0.0001
	$CuSO_4$ $5H_2O$	0.0001
	Na_2MoO_4 $2H_2O$	0.0001

Sterilised 15 mins. at 15 p.s.i.g

Solution 3	Biotin	0,10 or 20 μg/l
	Choline	0,1 or 2 mg/l
	Methionine	0 or 600 mg/l
	Sterilised separately by filtration	

All solutions were added, as necessary, aseptically to the medium reservoir then fed to the 8.5 litre chemostat with the following growth conditions: Temperature 30° C., aeration 1vvm; agitation 800 r.p.m., single 6-bladed disc turbine, 0.5D, in fully baffled fermenter. The fermenter pH was maintained at 5.0 by the automatic addition of sterile ammonia. Solution 3 was changed in composition at various times to determine μmax with the various additions such as biotin alone or biotin plus choline. Growth rate 0.1 to 0.15 hr^{-1}.

The five isolates have the following morphological characteristics.

Media	Potato - Sucrose Agar	Czapek Dox Agar (Oxoid)
	250 grams of potatoes washed and diced, placed in pressure cooker 15 lbs. square inch for 15 minutes. The decoction is then squeezed through two layers of muslin. 2% of Glucose and 2% of Agar are added to the turbid filtrate and the medium autoclaved and dispersed.	

Growth conditions: 25° C.

Character of growth

Isolate I—7

Colony morphology similar to parent I.M.I. 145425 except colony diameter is smaller, 1.5cm. in three days at 30° C. on C.D.A. The floccose aerial mycelium varies in degree from colony to colony but is less than I—8. Older cultures have a brown discoloration to the mycelium. Reverse, yellow-brown to greyish-rose sectors with some pigment diffusing.

Isolate I—8

Very intense white floccose aerial mycelium. Colony diameter again less than parent I.M.I. 145425, 2.0 cm in three days at 30° C. on C.D.A. Reverse salmon pink to greyish-rose segments.

Isolate I—9

Macroscopic appearance close to I—8 but reverse lighter, salmon to hyaline in colour.

Isolate I—15

Small restricted dome shape colonies. Mycelium short, tangled and with tendency to be convoluted. Many sporodochia-like structures formed giving pink appearance at point of inoculation of streak. Pink coloration at periphery. Colony diameter only 0.4cm at 3 days at 30° C. on C.D.A.

Isolate I—16

Isolate I—15 is very unstable and continually gives rise to I—16. This isolate has an appearance similar to I—7 although colonies tend to be slightly greater in diameter, 2.4 cm. in 3 days and more even in appearance. I—16 is more stable than I—15.

Conidia

Isolate I—7

Abundant macroconidia produced in similar fashion to parent I.M.I. 145425. Sporodochia formed in older cultures. Individual macroconidia similar to parent I.M.I. 145425 in shape and size, 25—50μ×3.0—5.0μ. In older cultures many sporodochia are formed. Chlamydospores are also abundant in the mycelium of this isolate, intercalary and terminal. They are globose smooth 10—12μ. Occasionally a single cell of a macroconidium forms a chlamydospore.

Isolate I—8

Fewer macroconidia than I—7 and those present mainly smaller and simpler, 1 to 3 septate, 25—35μ in length. Few sporodochia formed. Chlamydospores again abundant, intercalary, terminal, single and in chains.

Isolate I—9

Very similar to I—8 except more macroconidia, almost equivalent to I—7 in number.

Isolate I—15

Very few macroconidia, the sporodochia-type structures are in fact made up of packets of chlamydospores. There are also many chlamydospores present in the mycelium. The macroconidia are smaller than the parent, 30—35$\mu \times 4\mu$ with only 2 septa.

Isolate I—16

Very similar to I—7 with abundant macroconidia and chlamydospores. The macroconidia are typical, 30—45$\mu \times 4\mu$.

Examples 6 to 11 are examples of fermentation describing fungal cultures containing the parent strain *Fusarium graminearum* Schwabe I.M.I. 145425 and variants (or isolates) thereof in culture media.

EXAMPLE 6.

Duplicate shake flasks of 1-litre capacity were prepared containing 200 mls. of the following medium:

		Final concentration %
Solution 1	Glucose (sterilised separately pH 3.0, 10 p.s.i.g./10 min.)	3.0
Solution 2	Ammonium sulphate	0.565
	Potassium Dihydrogen Phosphate	1.0
	$MgSO_4:7H_2O$	0.025
	$FeSO_4:(NH_4)_2SO_4:6H_2O$	0.0005
	$MnSO_4:4H_2O$	0.0005
	$CuSO_4:5H_2O$	0.0001
	$CoCl_2:6H_2O$	0.0001
	$CaCl_2:2H_2O$	0.0015
	Na_2MoO_4	0.00001
	NaOH	0.2
	Salts sterilised at 15 p.s.i.g./15 min. Final pH 6.0	
Solution 3	Vitamins as described below were sterilised by filtration	

The solutions were added aseptically to give a final volume of 200 ml. then the flasks were inoculated with washed spores of our strain of *Fusarium graminearum* I—7 IMI 154209 to give a concentration of 8×10^5/ml.

The conditions of growth were temperature 30° C., 140 r.p.m. on orbital shaker with 2" throw.

At hourly intervals the growth was measured by measuring the Optical Density of a sample at 600 mμ. From the results obtained the following growth rates were established.

		Growth rate h^{-1}
(i)	Solution 3 omitted (minimal medium)	very slow
(ii)	Solution 3 such that the final concentration of Biotin in the culture medium was 50 μg/l	0.22
(iii)	Solution 3 such that the final concentration of Biotin in the culture medium was 50 μg/l and Choline chloride 50 mg/l.	0.27

EXAMPLE 7.

The procedure of Example 6 was repeated but the strain of I—7 was replaced by our strain of *Fusarium graminearum* Schwabe I—8. The following growth rates were established:—

	Growth rate h^{-1}
(i) As 6(i)	very slow
(ii) As 6(ii)	0.22
(iii) As 6(iii)	0.27

EXAMPLE 8.

The procedure of Example 6 was repeated but the strain I—7 was replaced by our strain of *Fusarium graminearum* Schwabe I—9. The following growth rates were established:—

	Growth rate h^{-1}
(i) As 6(i)	very slow
(ii) As 6(ii)	0.21
(iii) As 6(iii)	0.27

EXAMPLE 9.

The procedure of Example 6 was repeated but the strain I—7 was replaced by our strain of *Fusarium graminearum* Schwabe I—15. The following growth rates were established:—

	Growth rate h^{-1}
(i) As 6(i)	very slow
(ii) As 6(ii)	0.21
(iii) As 6(iii)	0.27

EXAMPLE 10.

The procedure of Example 6 was repeated but the strain I—7 was replaced by our strain of *Fusarium graminearum* Schwabe I—16. The following growth rates were established:—

	Growth rate h^{-1}
(i) As 6(i)	very slow
(ii) As 6(ii)	0.21
(iii) As 6(iii)	0.27

EXAMPLE 11.

The procedure of Example 6 was repeated but the strain I—7 was replaced by the parent strain *Fusarium graminearum* Schwabe IMI 145425. The following growth rates were established:—

	Growth rate h^{-1}
(i) As 6(i)	very slow
(ii) As 6(ii)	0.22
(iii) As 6(iii)	0.27

WHAT WE CLAIM IS:—

1. *Fusarium graminearum* Schwabe deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 145425 and variants and mutants thereof.

2. *Fusarium graminearum* Schwabe I—7 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154209.

3. *Fusarium graminearum* Schwabe I—8 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154211.

4. *Fusarium graminearum* Schwabe I—9 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154212.

5. *Fusarium graminearum* Schwabe I—15 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154213.

6. *Fusarium graminearum* Schwabe I—16 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154210.

7. Fungal cultures containing a strain of *Fusarium graminearum* Schwabe I.M.I. 145425 or a mutant or variant thereof in a culture medium in which this strain is present in a culture medium containing or being supplied with nutrients or additives necessary for the sustenance and multiplication of the strain, the medium having a pH between 3.5 and 7 and the temperature of the medium being maintained at a precise value within the range of between 25 and 34° C.

8. A method for cultivating a strain of *Fusarium graminearum* Schwabe I.M.I. 145425 or a mutant or variant thereof wherein the strain is present in a culture medium containing or being supplied with nutrients or additives necessary for the sustenance and multiplication of the strain, the medium having a pH between 3.5 and 7 and the temperature of the medium being maintained at a precise value within the range of between 25 and 34° C.

9. A method for the preparation of variants of *Fusarium graminearum* Schwabe I.M.I. 145425 which comprises growing the parent strain I.M.I. 145425 under continuous culture conditions with carbon limitation in a fermentation.

10. A method for the preparation of variants of *Fusarium graminearum* Schwabe I.M.I. 145425 which comprises growing the parent strain I.M.I. 145425 on a glucose based medium at 25° to 30° C. under continuous culture conditions at a dilution rate of 0.10 to 0.15 hrs.$^{-1}$ with carbon limitation in a fermentation for 1100 hours.

11. A method as claimed in claim 10 wherein the resulting proliferated variants are isolated by dilution plating.

12. A method for the preparation of variants of *Fusarium graminearum* Schwabe I.M.I. 145425 substantially as described with reference to Examples 1 to 5 hereinbefore set forth.

13. Fungal cultures containing *Fusarium graminearum* Schwabe I.M.I. 145425 or mutants or variants thereof substantially as described with reference to any one of Examples 6 to 11 hereinbefore set forth.

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(54) IMPROVEMENTS IN OR RELATING TO MICROORGANISMS

PATENTS ACT 1949

SPECIFICATION NO 1346061

The following amendments were allowed under Section 29 on 21 August 1978

Page 2, line 8, Page 3, line 18, *for* Glucose *read* Sucrose

THE PATENT OFFICE
19 September 1978

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The invention further provides the following variants:—

I—7 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154209.

I—8 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154211.

I—9 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154212.

I—15 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154213.

I—16 deposited with the Commonwealth Mycological Institute and assigned the number I.M.I. 154210.

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According to the invention in another aspect there are provided fungal cultures containing a strain of *Fusarium graminearum* Schwabe, I.M.I. 145425 (or a mutant or variant thereof) in a culture medium, and methods for cultivating the same strain, in which this strain is present in a culture medium containing or being supplied with nutrients or additives necessary for the sustenance and multiplication of the strain, the medium having a pH between 3.5 and 7 and the temperature of the medium being maintained at a precise value within the range of between 25 and 34° C.